PYRUVATE DEHYDROGENASE ACTIVITY IN RESPONSE TO SKELETAL MUSCLE CONTRACTION AT TWO STIMULATION FREQUENCIES IN PYRUVATE DEHYDROGENASE KINASE 2 KNOCKOUT MICE

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ABSTRACT

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Pyruvate dehydrogenase (PDH) plays an important role in regulating carbohydrate oxidation in skeletal muscle. PDH is deactivated by a set of PDH kinases (PDK 1-4) with PDK2 and 4 being the predominant isoforms in skeletal muscle. PDK2 is highly sensitive to pyruvate inhibition, and is the most abundant isoform, while PDK1 and 4 protein content are normally lower. This study examined the PDK isoform content and PDHa activation in muscle at rest and 10 and 40 Hz stimulation from PDK2 knockout (PDK2KO) mice to delineate the role of PDK2 in activating the PDH complex during low and moderate intensity muscle contraction. PDHa activity was lower in PDK2KO mice during contraction while total PDK actitvity was ~4 fold lower. PDK4 protein was not different, however PDK1 partially compensated for the lack of PDK2 and was ~56% higher than WT. PDK1 is a very potent inhibitor of the PDH complex due to its phosphorylation site specificity and allosteric regulation. These results suggest that the site specificity and allosteric regulatory properties of the individual PDK isoforms are more important than total PDK activity in determining transformation of the complex and PDHa activity during acute muscle contraction.

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Acetyl-CoA	acetyl-coenzyme A
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine sreum albumin
Ca^{2+}	calcium
CoA	coenzyme A
COX IV	cytochrome *c* oxidase IV
EGTA	ethylene glycol tetra acetic acid
E1-α	pyruvate dehydrogenase
E2	dihydrolipoamide transferase
E3	dihydrolipoamide dehydrogenase
ETC	elctron transport chain
FFA	free fatty acid
HIF-1	hypoxia-inducible factor 1
KO	knock-out
N_2	liquid nitrogen
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nictinamide adenine dinucleotide
PCA	perchloric acid
OAA	oxaloacetate
PDH	pyruvate dehydrogenase
PDHa	active pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PDP	pyruvate dehydrogenase phosphatase
TCA	tricarboxylic acid cycle
TPP	tiamine pyrophosphate

CHAPTER 1

INTRODUCTION

The utilization of carbohydrates and fat by skeletal muscle for energy is a very important and well documented process. The pyruvate dehydrogenase (PDH) complex is a major player in the oxidation of carbohydrates, specifically in the rate-limiting conversion of pyruvate to acetyl-CoA. This complex is extremely important as it links glycolysis to the tricarboxylic cycle (TCA cycle) by regulating the entrance of carbohydrate-derived acetyl units into the TCA cycle (Harris et al. 2002).

The PDH complex is regulated by two enzyme families, the PDH kinases (PDK1-4) and the PDH phosphatases (PDP1 and 2). The two families work in opposition to one another as the main role of PDK is to phosphorylate and deactivate the PDH complex, while PDP works to dephosphorylate and reactivate the PDH complex. PDK2 and 4 are the predominate isoforms in skeletal muscle, and are therefore believed to mediate the majority of PDH complex deactivation in this tissue. Additionally, the individual isoforms respond differently to intramitochondrial effector concentrations, adding another level of regulation (Harris et al. 2002) (see appendix for more individual kinase information). Therefore, the population of the different PDKs at any given time will help determine how the complex behaves with any acute intervention such as during dietary perturbations (Wu et al. 1998, Wu et al. 1999), exercise (Watt et al. 2002, Watt et al. 2004) or exercise training (LeBlanc et al. 2004).

The PDK2 isoform is the most ubiquitous and abundantly expressed isoform in all tissues, with especially high concentrations in the liver, heart, kidney and skeletal muscle (Sugden and Holness, 2003). PDK2 is highly sensitive to pyruvate inhibition (Bowker-Kinley et al., 1998), and is known to increase in skeletal muscle with exercise training

(LeBlanc et al., 2004), but is generally relatively unchanged with dietary perturbations such as starvation and high fat diets (Holness et al. 2000, Peters et al. 2001, Jeoung et al., 2006). This thesis will examine the role that PDK2 would normally play determining the activation of the PDH complex during low and moderate intensity muscle contraction through the examination of genetically modified mice that are missing (or "knocked out") the PDK2 gene. Using a knockout model has its limitations, because it has been genetically manipulated and might not reflect a physiologically accurate environment. In many cases, using this severe genetic approach can induce adaptations to compensate. Although this should be taken into consideration, using a knockout model allows isolation of the removed gene, and permits the analysis of the effect of its deletion.

CHAPTER 2

LITERATURE REVIEW

PDH Complex

a) Role in Metabolism

PDH plays a pivotal role in the regulation of glucose homeostasis by catalyzing the oxidative decarboxylation of pyruvate to acetyl-CoA. This reaction links glycolysis to the TCA cycle, as PDH controls carbohydrate oxidation by regulating the entrance of carbohydrate derived acetyl units into the TCA cycle. Consequentially, adequate flux through PDH is important for tissues with a high ATP requirement, including exercising muscle. Suppression of PDH activity is crucial for conservation of carbohydrates (and to facilitate fatty acid oxidation) when these are in short supply (Sugden and Holness, 2003).

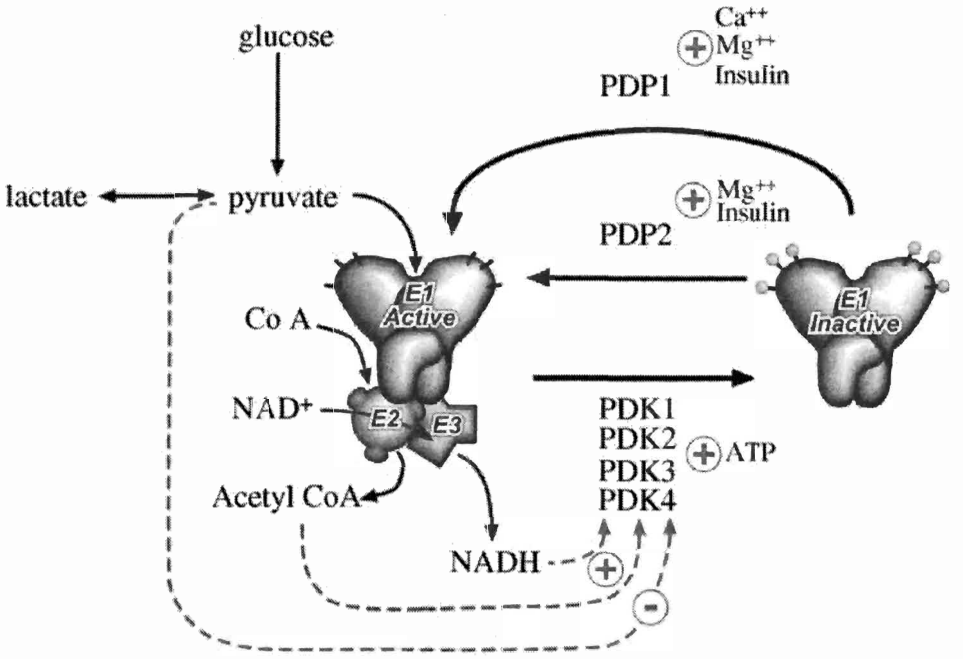

Figure 1. The regulation of PDHa activity through reversible phosphorylation. Relative degree of phosphorylation of the E1-alpha subunit is determined by kinases (PDK1–4) and phosphatases (PDP1 and 2). Regulators of phosphatases and kinases are highlighted. E1 is shown in blue, E2 is shown in green and E3 is shown in yellow. (Maj et al. 2006)

b) Structure of the Complex

The PDH complex is built around a 60 subunit inner core of tightly associated dihydrolipoamide acetyltransferase subunits (E2). Each E2 subunit has an inner region that forms the core and an outer region that extends from the core which binds the E1 enzyme. The E1 enzyme is responsible for the irreversible decarboxylation of pyruvate and each outer region consists of three self-folding domains connected in series. Dihydrolipoamide dehydrogenase (E3) is responsible for oxidation of E2 and the production of NADH in an FAD-linked reaction. E3-binding protein is not catalytic and only plays a structural role in the binding of E3 to the complex [Fig. 1]. It has been proposed that 48 subunits of E2 and twelve subunits of E3BP form the central core of the PDH complex (Harris et al. 2002, Patel and Korotchkina, 2006). The PDH complex also contains two families of regulatory enzymes, the PDH kinases (PDKs) of which there are four isoforms (PDK1-4), and the PDH phosphatases (PDPs) of which there are two isoforms (PDP1 and PDP2). Both enzymes catalyze a phosphorylation-dephosphorylation cycle which involves specific phosphorylation sites on the E1-α subunit (Wieland, 1983). The phosphorylation of the E1-α subunit renders the PDH complex completely inactive (Linn et al. 1969) and occurs at three specific serine residues (Yeaman et al. 1978). The three serine residues are serine-264 (designated site 1) serine-271 (designated site 2) and serine-203 (designated site 3) and have been designated as a result of the rate of phosphorylation (Yeaman et al. 1978). Through study of phosphorylation rates of the rat heart, it was determined that phosphorylation of site 1 is the most rapid while phosphorylation of site 3 is the least rapid (Sale and Randle, 1981). It was also determined that site 1 is the site of major inactivation in bovine kidney and is responsible for 60-70% of occurring inactivation, while sites 2 and 3 only add to the inactivation

(Yeaman et al. 1978). As such, phosphorylation of only one site renders this component inactive (Patel and Korotchkina, 2006), specifically, phosphorylation of site 1 is correlated with major inactivation, while increased occupancy of sites 2 and 3 renders the complex less sensitive to activation by PDP (Korotchkina and Patel, 1995, Korotchkina et al. 1995). The specific regulation of these enzymes will be discussed in greater detail later.

c) Regulation of the complex

i) Covalent and Allosteric regulation

Physiologically, the complex is rarely completely active or inactive and the proportion of collective complex activity is determined by the relative activities of the PDH kinases and the opposing PDH phosphatases (Harris et al. 2002).

The PDKs are regulated allosterically by the mitochondrial concentrations of pyruvate, ADP and Pi, and NADH/NAD^+ and acetyl-CoA/CoA ratios [Fig. 2]. Pyruvate is of particular importance as its concentration fluctuates markedly during different metabolic states and exercise conditions (Harris et al. 2002). High pyruvate levels inhibit PDK activity, upregulating the complex, while a high energy state, high levels of ATP, NADH, acetyl-CoA, increases PDK activity to down regulate the complex. NADH and acetyl-CoA, products of the PDH reaction, remain bound to the complex when they accumulate, which in turn affects the amount of PDK bound to E2. Binding of the PDK to an E2 domain results in higher kinase activity and an increase in PDH down-regulation (Patel and Korotchkina, 2006).

PDP1 requires Mg^{2+} but is allosterically stimulated by Ca^{2+}. Conditions that cause an increase in Ca^{2+} concentration, like muscle contraction, will stimulate the activity of

PDP1 and therefore the activity of the PDH complex. The second PDP isoform (PDP2) is not activated by Ca^{2+} and needs higher Mg^{2+} concentrations for activation. Therefore, it is presumably sensitive to nutritional perturbations, through changes in insulin concentration (Huang et al. 2003).

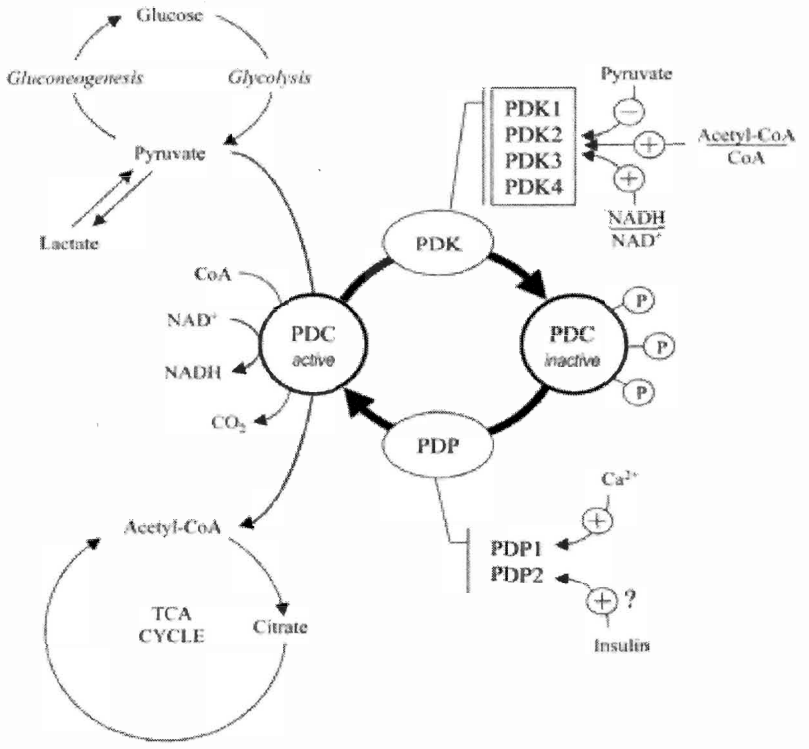

Figure 2. Regulation of the PDH complex. PDK - Pyruvate dehydrogenase kinase; PDP - Pyruvate dehydrogenase phosphatase; PDC- Pyruvate Dehydrogenase Complex (Sugden & Holness, 2003).

PDK 2 and 4

a) Discovery and Biochemical Properties

Early studies had found that high fat feeding over 19-23 days created a marked decline in PDHa activity without a change in total PDH activity (Fuller and Randle, 1984). The mechanism behind this response was studied earlier by Hutson and Randle (1978), who focused on the mitochondrial effectors for an explanation. The authors found that there was an increase in PDK activity in rat heart mitochondria from diabetic and 48h starved rats. The increase seen in PDK activity persisted even after the mitochondrial effectors had been washed away during mitochondrial extractions. Demonstrating that the response observed was unrelated to acute changes in mitochondrial effectors and was therefore termed a "stable increase" in PDK activity (Hutson and Randle, 1978).

Additional work to determine the reason for the stable increase in PDK activity centered around free PDK and/or an activator of the kinase termed the "kinase activator protein" (KAP) (Kerbey and Randle, 1982). KAP was found to increase during diabetes and starvation and the increase was reversed by re-feeding of the starved rats. However, both Mistry et al. (1991) and Jones and Yeaman (1991) simultaneously demonstrated that the KAP found in liver mitochondria was actually unbound kinase, therefore eliminating the concept of an activator protein for PDK.

More recent work discovered that there were four PDK isoforms (Priestman et al. 1994, Gudi et al. 1996, Rowles et al. 1996, Bowker-Kinley et al. 1998). The first successful attempt of purification of PDK reported that the PDK enzyme existed as two subunits of 48 and 45 kDa, designated as α- and β-subunits. Kinase activity was believed to reside solely in the α-subunit while the function of the β-subunit was unknown. It was suggested that the β-subunit might be involved in regulation. Priestman et al. (1994) first

suggested that the α-subunit was not the only active kinase, by demonstrating that antibodies directed against this subunit did not detect enough PDK protein to account for the full increase in PDK activity observed during starvation. Once it was determined that both the α and β proteins were catalytically active, it was clear that they were not subunits at all, but two different isoforms with different specific activity that were renamed PDK1 and 2 respectively (Popov et al. 1994, Priestman et al. 1994). Specifically, the primary structure of PDK1 was identified as a 48-kDa kinase while PDK2 was a 45-kDa kinase.

Gudi et al. (1996) uncovered the third PDK isoform in humans, which was called PDK3. Previously, most of the studies of the regulation of PDH activity had been performed on purified preparations of the PDH complex or the mitochondria prepared from different tissues with unspecified amounts of PDK which meant that the question of individual contributions of PDK isoforms had never really been addressed. These authors then compared the three isoforms using an ***in vitro*** ATP-dependent inactivation assay, which measured the rate of inactivation of PDH activity due to phosphorylation (Gudi et al. 1996). PDK3, another 45-kDa kinase, was found to be the most active isoform. The specific activity of PDK1 was about 30-50% lower than PDK3 while PDK2 had the lowest basal activity of all three enzymes (Gudi et al. 1996). However, these results could be misleading as the kinase activity rate ***in vivo*** depends upon the intramitochondrial concentrations of products and substrates of the dehydrogenase reaction.

The fourth PDK isoform was identified by Rowles et al. (1996). The authors studied Pima Indians, who have the highest known prevalence of type 2 diabetes, to determine the genetic sequence of the newly discovered PDK4 isoform. It was found that the PDK4 gene was associated with the gene defect which is prominent in Pima Indians and correlated strongly with the prevalence of type 2 diabetes.

Bowker-Kinley et al. (1998) provided evidence that the isoforms of PDK were responsible for tissue-specific regulation of PDH complex activity. The authors found that of the four isoforms studied, only PDK2 was ubiquitously and abundantly expressed, suggesting that PDK2 is the major isoform responsible for metabolic control over PDH. PDK4 was expressed in skeletal muscle and heart and when compared to PDK2 was shown to have a higher specific activity but a lower sensitivity to dichloroacetate (the synthetic analogue of pyruvate). PDK3 was the most unique because of its high specific activity and unique allosteric regulation. PDK3 showed little response to NADH, while the simultaneous presence of NADH and acetyl-CoA resulted in 50% of its inactivation (Bowker-Kinley et al. 1998). Its activity is the highest out of the four isoforms, and its expression is extremely low in most rat tissues except the testes. Finally, the role of PDK1 is the most unknown, as its tissue distribution is limited to the heart muscle, and oxidative skeletal muscle (Bowker-Kinley et al. 1998, Peters et al. 2001). PDK1 has a higher specific activity than both PDK2 and PDK4 and is even more sensitive to inhibition by dichloroacetate and ADP. The primary structure of the four isoforms is similar, with 66-74% identity, while between PDK1 and PDK2 there is 70% identity. The conclusions drawn by these authors centre around the idea that PDK1 and PDK2 isoforms are specialized for short-term/metabolic control of PDH activity, while PDK4 is more involved in the regulation of adaptive responses to nutritional changes such as starvation (Bowker-Kinley et al. 1998; Sugden et al. 2003).

b) PDK2 and PDK4 in Starvation, Diabetes and Following a High Fat Diet

The mechanisms of adaptive regulation of the PDH complex are not well understood and usually occur as the result of changes in enzyme activity and/or protein content. The most studied conditions surrounding the adaptive regulation of the PDH complex are food-restriction, high fat-diets and chemically-induced diabetes. These conditions involve a decrease in carbohydrate utilization and an increased reliance on free fatty acid (FFA) metabolism as well as increased beta-oxidation. An increase in PDK activity will cause a functional shift in PDH from the active form to the inactive form through enhanced phosphorylation (Harris et al. 2002; Sugden et al. 200; Peters et al. 2001; Wu et al. 1999). In more acute situations there is an increase in beta-oxidation and both the $NADH/NAD^+$ and acetyl-CoA/CoA ratios can regulate the complex.

One of the first studies to provide an explanation for this mechanism tested the hypothesis that starvation and diabetes may increase the amount of one or more of the PDK isoforms in the rat heart (Wu et al. 1998). After 48 h starvation, a 2-3 fold increase in PDK activity was found, with a greater than 3-fold increase in the amount of PDK4 protein, while PDK2 was unaltered. Streptozotocin treatment was used to induce a type 1 diabetes-like state by destroying the insulin-producing beta cells of the pancreas. A stable increase in PDK activity was also seen after this treatment, with a significant increase in PDK4 protein content and no change in PDK2 protein content. The authors then tested the diabetic rats to see if the effects on PDK activity and PDK4 expression could be reversed by insulin. The treatment of the diabetic rats with insulin decreased the level of PDK4 protein almost to the level seen in the control rats. This demonstrated that a large increase in the amount of rat PDK4 protein correlated with the stable increase in PDK activity normally seen in starved and diabetic rats. The magnitude of the PDK isoform

shift induced by starvation and diabetes appears to be almost entirely accounted for by PDK4 protein expression (Wu et al. 1998).

These same investigators examined rat skeletal muscle to see if there would be a similar increase in the PDK4 isoform (Wu et al. 1999). The authors hypothesized that the increase in long-chain fatty acids caused by starvation and chemically induced diabetes would increase PDK4 expression through activation of the peroxisome proliferator-activated receptor-α (PPAR-α) which is a transcription factor that regulates the expression of certain genes. PDK activities from mitochondria isolated from skeletal muscle (mixed gastrocnemius) were shown to increase nearly three-fold following the 48-h starvation and nearly four-fold with streptozotocin-induced diabetes. It was found that PDK2 and PDK4 were expressed in significant amounts in rat skeletal muscle with these perturbations. Feeding the rats the known agonist for PPAR-α (WY-14, 643) mimicked the effect of starvation and diabetes on PDK4 expression causing 3-5 fold increases in this isoform in the gastrocnemius muscle. This confirmed that PPAR-α could be stimulated by fatty acids and was at least one mechanism to increase expression of PDK4 (Wu et al. 1999).

Skeletal muscle is not homogenous, there are three types of fibres which are organized based on their contractile capabilities: type I (slow-twitch oxidative), type IIA (fast-twitch oxidative glycolytic), and type IIB (fast-twitch glycolytic) with the balance made of transition fibers (type IIX/IID) (Delp and Duan, 1996). In the fed state, oxidative slow-twitch muscles have higher glucose utilization rates than fast-twitch muscle fibres, which tend to be recruited during exercise (Putman et al. 1993).

Peters et al. (2001) examined PDK activity and PDK isoform protein changes in rat soleus (84% type I, 7% type IIA, 0% type IIB) red gastrocnemius (30-51% type I, 35-

62% type IIA, 1-8% type IIB) and white gastrocnemius (0% type I, 0% type IIA, 92% type IIB) muscles over a 24 h fasting period (Peters et al. 2001). Rat skeletal muscle only has measureable quantities of the PDK 1, 2 and 4 isoforms and this study was the first to examine the PDK activity changes in all three PDK isoforms. The authors found that the fast twitch-glycolytic fibres differed from the oxidative fibers in PDK activity. There was an increase in PDK activity in all muscle types but the fast-twitch glycolytic fibres had 50% lower PDK activity in both fed and fasted rats with the changes in PDK4 protein expression corresponding to these results. PDK2 protein expression was not different between fibre types and was not affected by the 24 h fast (Peters et al. 2001).

This work was further explored by Sugden et al. (2000) with a longer starvation period of 48 h, who demonstrated, using rat soleus and tibialis anterior (66% type IIA, 32% type IIB, 2% type I) there was a difference in the up-regulation of PDK2 compared to PDK4 between fibre types. They examined pyruvate sensitivity after starvation in the two skeletal muscle types and found that as PDK4 content increased, PDHa activity became more insensitive to pyruvate activation, confirming what had been predicted from the ***in vivo*** studies. They concluded that PDK4 was targeted for up-regulation during starvation and other dietary perturbations specifically because it made the complex more insensitive to pyruvate to divert the 3-carbon units to liver glucose production and away from oxidation (Sugden et al. 2000). In rats, high-fat feeding results are similar to the adaptations of food-restriction; however, 28 d of high-fat feeding were necessary to elicit the same changes in PDK activity as was seen with only 48 h of food-restriction (Orfali et al. 1993, Holness et al. 2000).

Although work on PDK activity and gene expression had been explored in the rodent model, little was known about whether a similar response would be observed in human

skeletal muscle. Peters et al. (1998) hypothesized that PDK activity would increase on a low carbohydrate diet and this would be accompanied by a decreased PDHa activity. The authors found a dramatic 3-5 fold increase in PDK activity in human skeletal muscle after the low carbohydrate dietary intervention after only 3 days. Decreased insulin and increased free fatty acid concentrations were thought to be potential regulators of the stable PDK increase (Peters et al. 1998).

Peters et al. (2001) then examined the protein and mRNA expression of PDK2 and 4 over these first 3 days of the low carbohydrate high fat diet. They found that with an increase in PDK activity there was a parallel increase of PDK4 protein, while PDK2 protein was unchanged throughout the first three days. The increase in PDK4 protein was maximal within the first day of the dietary intervention, and since there was a linear increase of PDK4 activity over the six days, this suggests that perhaps another PDK isoform contributed to the continuous increase in PDK activity or there was a change in the specific activity of the existing PDK2 or PDK4 isoforms. One of the possibilities for this change in activity is that the newly synthesized PDK4 or existing PDK2 improves its specific activity as it is bound to the PDH complex core (Peters et al. 2001). Another major finding by these authors was that there was a strong correlation between a subject's aerobic capacity and the rate of change in PDK activity in the first three days of the diet. However, this correlation disappeared by day six. It is possible that the more aerobically trained individuals were more sensitive to the decrease in insulin levels which resulted in the early increase in PDK activity (Peters et al. 2001).

Putman et al. (1993) examined the importance of acetyl group accumulation in PDH regulation in human skeletal muscle. Putman et al. (1993) used glycogen-depleting exercise followed by a low-carbohydrate diet compared to a high-carbohydrate diet

perturbation to shift from either fat to carbohydrate utilization. Muscle-depleting exercise was completed by the subjects who then consumed either a high-carbohydrate diet or a low-carbohydrate diet for three days. Activation of the PDH complex throughout exercise at 75% VO_2 max to exhaustion was attenuated following the low-carbohydrate high fat diet suggesting that dietary manipulations can compromise one's ability to fully activate PDHa and allow for carbohydrate oxidation (Putman et al. 1993).

However, initial muscle glycogen content was different between trials in that study, as the low-carbohydrate group had lower muscle glycogen levels at rest and during exercise than the high-carbohydrate group and glycogen utilization rates were lower following the high fat diet. To account for this difference, St. Amand et al. (1999) conducted a similar study with focus on maintaining glycogen content by eliminating the glycogen-depleting exercise, having a mixed diet group instead of the high carbohydrate group, and asking subjects to refrain from physical activity during the dietary intervention. The authors found that it was still very difficult to maintain glycogen content between the two diet perturbations, even without glycogen-depleting exercise, as the low carbohydrate diet still resulted in lower initial muscle glycogen. However, the difference in glycogen utilization during exercise was consistent across all time intervals for both the control diet and the high fat diet, and muscle pyruvate concentrations were similar. Therefore, PDHa activity increased during exercise to a similar extent, demonstrating that in spite of the fact that the pyruvate insensitive PDK isoform would be higher after the high fat diet, the increased pyruvate production was able to overcome the higher PDK activity (St. Amand et al. 1999).

c) Changes in PDK2 and 4 with Prolonged Exercise and Exercise Training

Prolonged exercise is characterized by a shift from carbohydrate as the main fuel source to increased fat utilization (Watt et al. 2004) with the PDH complex playing a major role in controlling the flux of carbohydrate oxidation. Pilegaard et al. (2000) studied the transcription rate and the mRNA responses in human skeletal muscle following 4 hours of cycling exercise at 50% maximal O_2 uptake. Through muscle biopsies, it was found that PDK4 mRNA and transcriptional rate increased after the 4 h period. The authors then concluded that PDK4 expression progressively increases with exercise duration which could contribute to the progressive inactivation of PDHa. However, the authors only measured mRNA content and transcription rate; they did not measure the amount of protein, total PDK activity or total PDHa activity (Pilegaard et al. 2000). Watt et al. (2004) conducted a similar study measuring PDK2 and 4 proteins to see whether changes in PDHa activity were due to increased gene expression of the isoforms. PDHa activity was decreased and PDK activity was modestly increased, but PDK2 and 4 protein concentrations remained unchanged after the 4 h exercise period. Therefore, the higher PDK activity was not caused by changes in PDK2 or 4 proteins and suggested that there could be an upregulation of specific activity of the existing kinases (Pilegaard et al. 2000, Watt et al. 2002, Watt et al. 2004).

LeBlanc et al. (2004) examined the adaptive responses to repeated exercise training. The authors had subjects train on a cycle ergometer at a power output of 75% of their maximal O_2 uptake for 1h per day, five days a week for a total of 8 weeks. After 1 week of exercise, neither total PDH nor PDK activity changed, but by week 8 total PDH activity had increased by 31% while PDK activity had almost doubled. During week 1 there was also no change in PDK protein expression and by week 8 only the PDK2

isoform had increased 1.3-fold. This study suggests that longer-term alterations to energy metabolism and energy status favour an up-regulation of PDK2, possibly making this the 'energy status'-responsive PDK isoform. It is thought that the training-induced adaptive increase in skeletal muscle PDK2, which is much more pyruvate sensitive than the other isoform, may be used to regulate PDH activation during sub-maximal exercise after training to promote increased down-regulation of the complex and enhance fat oxidation, but allow for increased activation of the complex, when necessary, with higher intensity exercise and increased glycolytic flux (LeBlanc et al. 2004).

Physiological Changes of the PDK1 Isoform

Currently, there is very little known about the PDK1 isoform, but it is present in high levels in the heart, with lower levels found in skeletal muscle, liver and pancreas (Patel and Korotchkina, 2006). Its catalytic activity is second only to that of the PDK3 isoform which is found in such small quantities in rodent tissue that it has yet to be accurately measured (Gudi et al. 1995, Bowker-Kinley et al. 1998). The PDK isoforms differ in their E1-α phosphorylation site specificity, and while all four PDK isoforms have the ability to phosphorylate sites 1 and 2, only the PDK1 isoform can phosphorylate site 3 (Korotchkina and Patel, 2001, Kolobova et al. 2001). Therefore, PDK1 has the ability to make the complex more difficult to dephosphorylate or activate by the PDPs.

Peters et al. (2001) were the first to examine the changes in PDK1 protein concentrations in three skeletal muscle fibre types. The expression of the PDK1 isoform was found to be much lower in glycolytic muscles compared to oxidative muscle, and PDK1 protein remained unchanged in response to a 24 h fast (Peters et al. 2001). The

mRNA expression of all four PDK isoforms in human skeletal muscle tissue after both 15 and 40 hrs of fasting was consistent with these data from rodents showing the PDK1 isoform to remain unaffected (Spriet et al. 2004). In human skeletal muscle the mitochondrial protein concentrations of PDK1 and 4 were found to be only ~10 % of the PDK2 content, which is abundantly expressed (LeBlanc et al. 2004). Although there was an increase in PDK2 protein after both 1 and 8 wks of exercise training, there was no change seen in the small amount of PDK1 protein (LeBlanc et al. 2004). These results consistently demonstrate that the PDK1 isoform is unaffected by perturbations that have shown to alter the expression of both PDK2 and 4. New information using total RNA prepared from human (abdominal muscle) rat (heart, abdominal muscle, spleen, liver brain, kidney, testis and lung) and mouse (liver and C_2C_{12} cells) (Huttemann et al. 2001) and cultured mouse and human (Hep3B, HeLa, Hct116 and A594) cells (Fukuda et al. 2007), regarding the effects of hypoxia, has shown that during low oxygen conditions, the PDK1 isoform has been shown to be upregulated by hypoxia-inducible factor 1 (HIF-1) (Semenza, 2007).

PDK2 and PDK4 Knockouts

The term "knockout" is used to explain the deletion or inactivation of a gene in a cell in order to determine what role the gene plays in the organism as a whole. Jeoung et al. (2006) created and tested these "knockout" mice for the PDK4 gene (PDK4 KO). The mice were lacking PDK4 in order to better understand the role that this protein normally played in PDH complex regulation with nutritional challenges such as prolonged starvation. Starvation for 48 h induced an increase in PDK4 protein content of wild-type

(WT) mice, as expected, but it was shown to have no effect on the already large amount of PDK2 protein in PDK4 KO mice. Therefore, there was no evidence found for the up-regulation of PDK2 to compensate for lack of PDK4 in diaphragm, while in gastrocnemius muscle of starved PDK4 KO mice, the amount of PDK2 protein was actually lower than in wild-type mice. The PDH complex was more active in the diaphragm of starved PDK4 KO mice (ie. higher PDHa activity), compared to starved wild-type, indicating that PDK4 would normally play a stronger role in down-regulation of the complex during starvation, while total PDK activity was not measured (Jeoung et al. 2006). Higher PDHa activity suggests that in PDK4 KO mice during starvation there is an increase in the oxidative disposal of glucose, through increased flux through the PDH complex, which would positively affect whole body glucose homeostasis.

In a more recent study, PDK4 KO mice were used to study glucose homeostasis in diet-induced obesity compared to WT mice, to establish the role of PDK4 in the obesity-induced hypoglycaemia by promoting increased muscle glucose disposal (Jeoung et al. 2006). Both WT and PDK4 KO mice were fed a high-fat diet (16% protein, 59.5% fat, 24.5% carbohydrate) for 18 weeks in order to induce obesity, while control groups for both PDK4 KO and WT mice were fed a chow diet. As expected, high-fat feeding caused obesity, fasting hyperglycaemia, increased fasting serum insulin levels, fasting glucose intolerance and insulin resistance in the WT mice. There was also an increase in PDK4 expression in gastrocnemius of WT mice which is consistent with previous studies involving the short-term effects of a high-fat diet (Holness et al. 2000). PDK4 KO mice also became obese from high-fat feeding and showed similarly elevated insulin levels. However, they had significantly lower fasting blood glucose levels and slightly improved glucose tolerance and insulin sensitivity. This study demonstrates that the absence of the

PDK4 isoform improved the negative effects of diet-induced obesity, promoting lower blood glucose and improved glucose tolerance suggesting that PDK4 could be a target for therapeutic models of diabetes.

Recently in our lab PDHa activity was examined at rest and at the onset of low and high intensity exercise conditions in incubated mouse extensor digitorum longus (EDL) muscles from PDK4 KO and WT mice (Martin et al. 2007). PDHa activity was higher with increased muscle stimulation intensity in both PDK4 KO and WT mice, but PDHa was higher at rest and during contraction at intensities of 10 and 40 Hz in PDK4 KO mice [Fig. 3]. In fact, at 40 Hz stimulation, which evoked ~50% PDHa activation in WT, PDHa was almost fully activated in PDK4 KO. This demonstrates that in the absence of PDK4, even though there may be the same amount of PDK2 present, the remaining kinase activity cannot appropriately modulate PDHa activity and implicates a role for PDK4 in 'down regulating' or 'fine-tuning' the activation of the complex during muscle contraction under normal conditions.

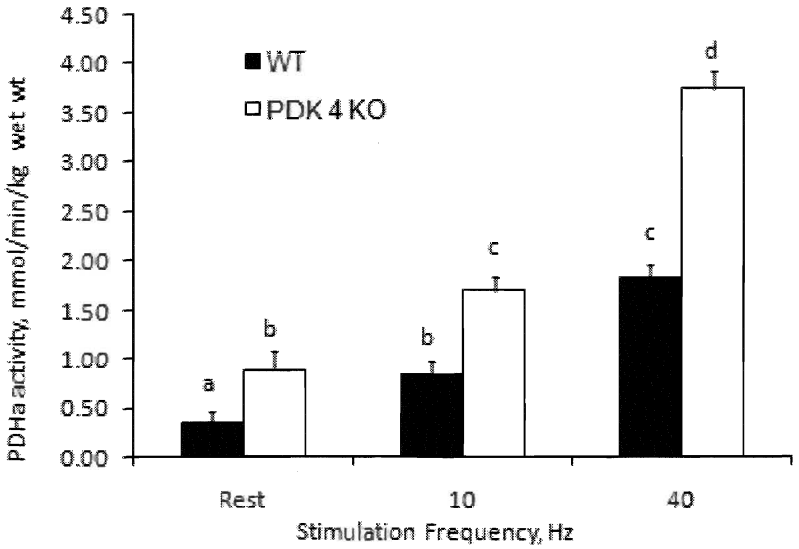

Figure 3. Previous work examining PDHa activity in incubated EDL muscles in wild-type mice and PDK4 knockout mice (PDK4KO) at Rest and at 10 Hz and 40 Hz stimulation. PDHa activity was higher in muscles from PDK4KO mice at Rest and both contraction intensities (Martin et al. 2007).

SUMMARY

PDK2 is the most abundant PDK isoform in skeletal muscle, and allosterically it is the most sensitive isoform to intra-mitochondrial pyruvate concentrations. The main role of PDK2, and other PDKs, is to phosphorylate and inhibit the PDH complex. In general, PDK2 expression is relatively unchanged with dietary challenges such as starvation and a high fat diet, both of which have shown to increase the expression of PDK4. However, with exercise training, when the reliance on fat is preferable, it is PDK2 that increases rather than PDK4. These results, as well as the high sensitivity of PDK2 to pyruvate concentrations, suggest that PDK2 plays an important role in decreasing PDHa activity during exercise after training and promoting fat oxidation, while allowing for PDH activation when needed, such as with an increased workload. However, the role that PDK2 plays in activating the PDH complex during muscle contraction is currently unknown.

CHAPTER 3

Statement of the Problem

1. Currently there is limited research on the most abundant PDK2 isoform and the subsequent effect of the deletion of this gene on the up regulation and activity of the other two skeletal muscle-predominant PDK isoforms 1 and 4 and PDH components E1-α and E2.

2. The role that PDK2 plays in regulating PDHa activity during an acute bout of muscle contraction is unknown.

Purpose

The purpose of this study was to determine the role that PDK2 plays in the regulation of PDHa activity at rest and during low intensity and moderate intensity muscle contraction in isolated skeletal muscle from PDK2 KO mice compared to their WT counterparts.

Hypothesis

The absence of the most abundant PDK2 isoform would result in an increase in PDHa activity in PDK2 KO mice when compared to WT at rest and during both stimulation conditions, with possible upregulation of PDK4 as a compensatory strategy. It was not expected that ablation of PDK2 would alter the PDH subunits.

CHAPTER 4

METHODOLOGY

Animals

Twenty-two C57BL/6J mice (11 PDK2 knockouts and 11 of their WT littermates) generously supplied by Dr. Robert Harris (Dept of Biochemistry and Molecular Biology, Indiana University School of Medicine) were used for this experiment. PDK2 KO and WT mice weighed 27 ± 5g and 25 ± 2g respectively and ranged in age from 9 – 12 months, both WT and PDK2 KO were age matched, one WT and one PDK2 KO experiment was performed simultaneously. The animals were housed in the Brock University Animal Care Facility for at least one week prior to being used in our studies in a controlled environment with a 12:12 h reversed light-dark cycle to be sure that they are fully acclimatized to their new surroundings. Animals were fed Purina Rat Chow (Ralston Purina Co.) *ad libitum* and were not fasted. All experimental interventions were formally approved by the Brock University Animal Care and Utilization Committee and conformed to all of the Canadian Council for Animal Care guidelines (AUPP 05-12-01).

Experimental Protocol

All animals underwent the same surgical and bath incubation protocols, but seperate incubations for muscle phosphagen determination and PDHa activity were completed. Animals were anesthetized with an intreperitoneal injection of sodium pentobarbital (6 mg / 100 g body wt.) and EDL and hindlimb muscles were removed. The hindlimb muscles were freeze-clamped within 10 s of their removal, while each EDL muscle had a suture placed on each tendon and then was immediately placed in an organ bath which was filled with fully oxygenated liquid Sigma medium 199 (M4530, see

appendix for detailed composition) and suspended at a resting tension of 1g of force. Muscles were allowed to incubate at rest for 30 min to equilibriate [Fig 4.]. After the initial incubation, the muscles were either removed within 10 s and freeze-clamped (rest) or stimulated with tetanic contractions every 5 s for a duration of 3 min at 10 Hz (low intensity) or 40 Hz (moderate intensity), then removed within 10 s and freeze-clamped for further analysis [Fig 5.].

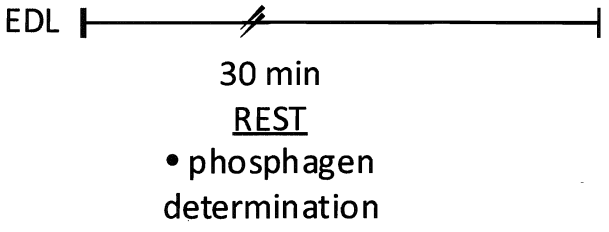

Figure 4. Schematic diagram of muscle phosphagen protocol. EDL muscles were excised and placed in an organ bath. The muscle was incubated for 30 min at rest. Muscles were freeze-clamped (<10s) and used for phosphagen determination.

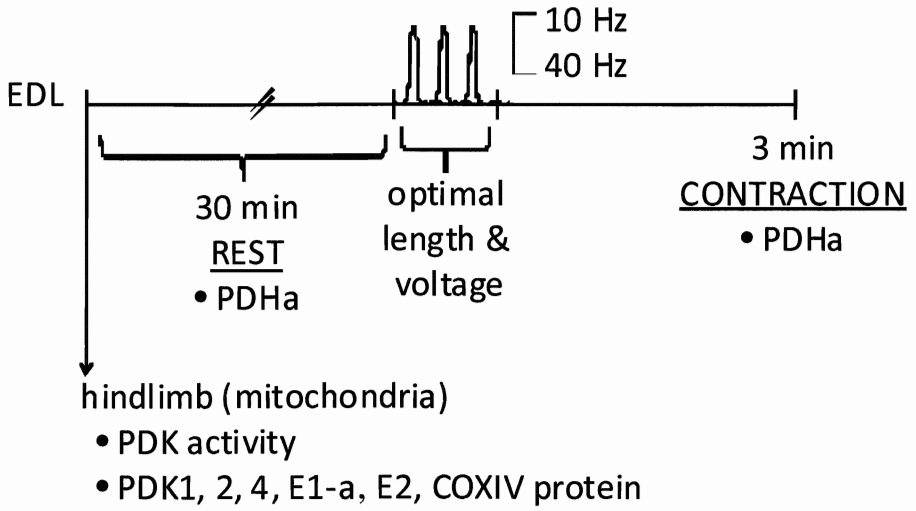

Figure 5. Schematic diagram of stimulation protocol. EDL and hindlimb muscles were excised. Mitochondria from the hindlimb was used for PDK activity and Western blotting for the PDKs, PDH components and COXIV protein determinations. EDL muscles were placed in an organ bath and attached to a transducer. Optimal length and voltage were determined after the muscle was incubated for 30 min at rest. Resting muscles were freeze-clamped (<10s) and used for PDHa activity determination. Muscles not used for rest went through the 3 min stimulation protocol at either 10 or 40 Hz. Muscles were freeze-clamped (<10s) and used for PDHa activity determination.

Force Acquisition

Optimal Stimulus Voltage and Optimal Length Determination

Before the stimulation protocol began, optimal stimulus voltage and optimal length (L_o) were determined by a force transducer (Grass Model FT03 with P11T amplifier). To establish optimal stimulus voltage, the muscle was suspended at 1g of resting tension and twitched, starting at 10 V with continuing single twitches until a plateau in force production was observed (~50-70 V).

Stimulation Protocol

The stimulation protocol consisted of a 200 ms train duration, a train rate of 0.2 trains per second and a stimulation frequency of 10 Hz or 40 Hz. Total contraction time was 3 min. All contraction data was recorded using a Grass Polyview Data Acquisition and Analysis System (West-Warwick, RI) and analyzed using Polyview Reviewer (Grass Polyview Data Acquisition and Analysis System; West-Warwich, RI).

Establishing a Force-Frequency Curve

Low and moderate stimulation was chosen to elicit a range of PDHa activities that would be ~50% of maximal PDHa activity. Pilot experiments were completed to establish a force-frequency curve [Fig. 6] (Martin et al. unpublished results) used to determine the frequencies required for low and moderate stimulation in relation to PDHa activity. Muscles from male C57BL/5J (Charles River, St. Constant, PQ) mice, (n = 5) were stimulated from 1-40 Hz and these frequencies were plotted against force production relative to maximal force, which was achieved at 150 Hz (F/Fmax). A frequency of 10 Hz was able to stimulate the muscle to approximately 20% of its maximal force, and was therefore selected as the low intensity frequency. However, a frequency of 40 Hz stimulated the muscle to contract at approximately 55% of its maximal force, and therefore was selected as the moderate intensity frequency.

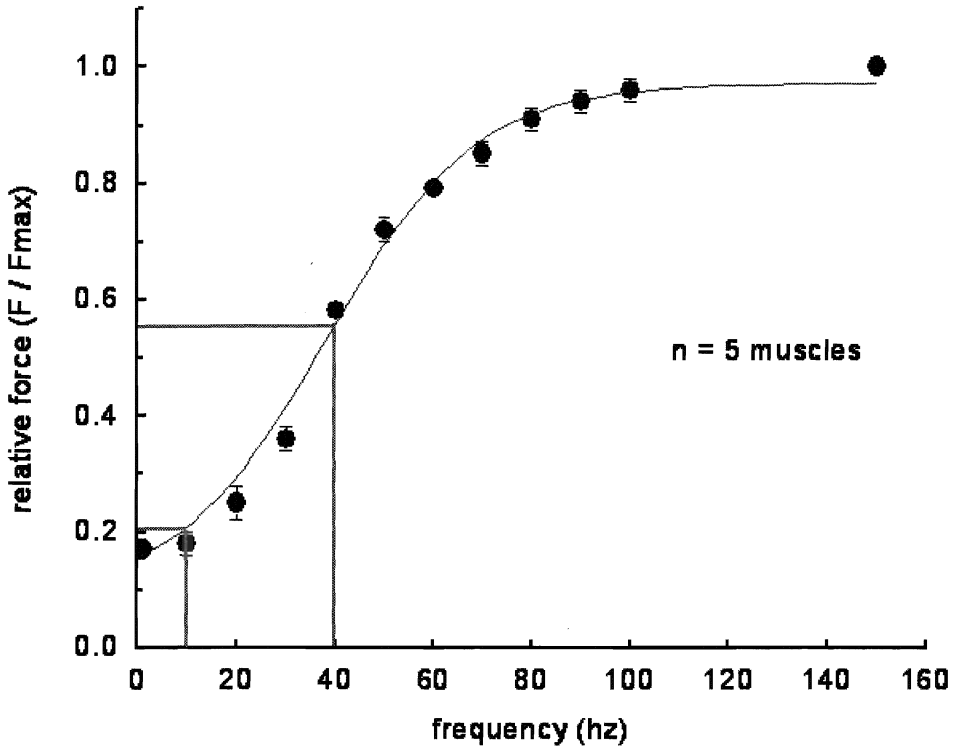

Figure 6. Force-frequency curve. Relative muscle force produced at various stimulation frequencies (Martin et al. 2007).

Mitochondrial Extraction

Intact mitochondria were extracted from mixed mouse hindlimb muscle by differential centrifugation as previously described by Jackman et al. (1996) and Makinen et al. (1968). Minced muscle was homogenized using a glass-on-glass Potter homogenizer in a buffer 20 vol of a buffer containing 100mM KCl, 40mM Tris-HCl, 10mM Tris base, 5mM magnesium sulfate, 1mM EDTA, and 1mM ATP (pH 7.5). The supernatant was retained after centrifugation at 700 *g* for 10 min and spun at 14000 g for 10 min. The supernatant was removed, but discarded, leaving the mitochondrial pellet which was washed, resuspended and centrifuged twice (7000 *g*) in 10 vol of 100mM KCl,

40mM Tris-HCl, 10mM Tris base, 1mM magnesium sulfate, 0.1mM EDTA, and 0.25mM ATP (pH 7.5). The first wash buffer included 1% bovine serum albumin, and the second was protein free. The final mitochondrial pellet was resuspended in a volume corresponding to 1 µl /1 mg fresh muscle extracted. The final buffer contained 220 mM sucrose, 70 mM mannitol, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.4). All procedures were completed at 0-4°C.

PDHa Activity

PDHa activity was measured according to Constantin-Teodosiu et al. (1991) as modified by Putman, et al. (1993) The homogenizing buffer to measure PDHa activity (PDH in the active form) containing 200 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM ethylene glycol tetraacetic acid (EGTA), 50 mM Tris HCl, 50 mM NaF (to inhibit the PDH phosphatase reaction), 5 mM dichloroacetate (to inhibit the PDH kinase reaction) and 0.05% Triton X-100 (pH 7.8), was placed into a homogenizing mortar (100 µl) and tared. Frozen muscle (3-10 mg wet) was added to the mortar, and its weight was recorded. Additional buffer was added to dilute the muscle to 30 volumes. The homogenization of the sample was initiated with gentle twisting of the pestle by hand. All homogenization was done with the sample on ice, and the movements were not hard enough to cause "bubbles" which could denature proteins and inactivate the enzyme. Grinding continued with a motor driven glass pestle for 30 s at ~20-30 rpm, also on ice. The homogenized sample was stored in liquid N_2 in an eppendorf tube prior to PDHa activity determination.

PDHa activity was measured as the rate of acetyl-CoA production. Muscle homogenate (30 µl) was added to720ml of reagent mixture containing 144.4mM Tris,

0.72mM EDTA, 1.44mM $MgCl_2$, 39 mM NAD, 13mM CoASH, 13mM TPP at 37°C in a 12x75 glass tube warmed in a block heater. The reaction was initiated with 26mM pyruvate (30 µl of 1mM solution) and 200 µl aliquots was removed at precisely timed intervals (1, 2, and 3 min) and placed into 40 µl of 0.5N perchloric acid (PCA) to stop the reaction. Samples were neutralized with 10 µl of 1M potassium carbonate (K_2CO_3) after 5 min following reaction with PCA. The neutralized extracts were stored at –80°C for analysis of acetyl-CoA concentration.

Acetyl-CoA concentration was measured in timed samples as outlined by Cederblad et al (1990) according to the citrate synthase reaction with radioactively labeled oxaloacetic acid to produce labeled citrate. Acetyl-CoA standards were prepared (0, 40nM, 125nM, 250nM, 375nM and 500nM) and 10 µl of each sample was added to 200 µl of water. The ^{14}C-oxaloacetic acid (OAA) substrate needed for the reaction was prepared according to the glutamate oxaloacetate transaminase reaction (GOT) (^{14}C-oxaloacetate + glutamate $\xrightarrow{\text{GOT}}$ ^{14}C-aspartate + 2-oxyoglutarate) 14C-OAA was added to the samples and the reaction was started with citrate synthase and allowed to proceed at room temperature for 20 min. N-ethylmaleimide (NEM) (30 mM) was added to bind to the ^{14}C-citrate produced and promote the forward citrate synthase reaction. ^{14}C-citrate produced in the reaction was separated from ^{14}C-oxaloacetate as follows: ^{14}C-oxaloacetate was converted back to ^{14}C-aspartate through the reverse transamination reaction. The resultant ^{14}C-aspartate was separated from ^{14}C-citrate through mixing with acidic Dowex resin (1.0 ml) and centrifuged (Beckman-Coulter Allegra 21R; Fullerton CA) at ~3000 rpm for 2 min. An aliquot of the supernatant containing ^{14}C-citrate was pipetted into scintillation vials and counted using a liquid scintillation counter (Beckman-Coulter LS; Fullerton, CA).

PDK Activity

Fresh skeletal muscle, from previously extracted mitochondria, was used and homogenized. The final pellet was resuspended in a 6-fold carbonylcyanide m-chlorophenylhydrazone (CCCP) buffer containing, 20 mM Tris-HCl, 120 mM KCl, 2 mM EGTA, 5 mM potassium phosphate (KH_2PO_4) to decrease ATP concentration to zero, resulting in compete conversion of PDH to the active form. This mixture was incubated at 30°C for 20 min, and then the mitochondria was pelleted at 7000 g for 10 min to pellet down the portion of the mitochondria containing the activated PDH complex. The top of the eppendorf tube containing the pelleted mitochondria was perforated and flash frozen into liquid N_2 to be later assayed for PDK activity.

On the day of analysis, the pellet was thawed and completely resuspended in 30 mM KH_2PO_4, 5 mM, EGTA, 5 mM, DTT, 1% bovine serum albumin (BSA), 1 mM tosyl-lysyl-chloro-methyl-ketone (protease inhibitor) 0.1% Triton, and 2 µl / 1 mg oligomycin B (F1-ATPase inhibitor) for a total of 1.0 ml. The suspension was freeze-thawed once more to ensure that the mitochondria were broken, and then kept in liquid N_2 until analysis. Magnesium ATP was added to the remaining suspension, bringing the concentration to 0.3 mM, and timed samples were taken every 30 s for 4 min at 30°C as previously described Fatania et al. (1986) and Vary et al. (1991).

PDK activity was measured as the apparent first-order rate constant of the inactivation of PDH (min^{-1}), or the slope of ln[%(PDH_a activity with ATP addition)/(total PDH without ATP addition)] vs. time. The slope (1^{st} order rate constant) was determined by a regression analysis of the line.

Western Blotting

All Western blotting was performed on extracted mitochondria as previously described (LeBlanc et al. 2004). Protein concentrations were measured using a Bio-Rad Protein Assay Reagent (BioRad, Hercules, CA) and detected on a microplate reader at 595nm wavelength (Biotek Instruments, Winooski, VT, USA).

Mitochondria were diluted to final protein concentration of 1.0 µg / µl in 50 mM Tris-HCL, pH 6.8, containing 2% SDS, 0.1 M dithiothreitol, 0.1% bromophenol blue, 10% glycerol, and a protease inhibitor cocktail (1 mM benzamidine, 0.1 mg/ml trypsin inhibitor, 0.1 mM tosyl-lysyl-phenylmethlketone) as previously described by Peters et al. (2001). Samples were then solublized by boiling for 5 min and then cooling for 5 min. Standard SDS-PAGE electrophoresis was performed with 12% separating and 4% stacking gels for all mitochondrial samples, PDK1, 2, 4, COX IV as well as PDH component determinations; E1-α and E2 (10 µg of mitochondrial protein per lane). Proteins were separated through electrophoresis and immersed in a running buffer containing 250mM Tris base, 1.92M glycine, and 0.1% SDS. The proteins were then transferred onto polyvinylidene fluoride (PVDF) (Millipore Corporation; Billerica, MA, USA) using the tank method of electrophoretic transfer (Bio-Rad, CA, USA) with a transfer buffer containing 34.8 mM Tris base, 31.2 mM glycine, 0.03% (w/v) SDS, and 20% (v/v) absolute methanol as previously described (LeBlanc et al., 2004). Membranes were then incubated in TBST buffer (20 mM Tris base, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5) and 5% dry milk for 1 h to block all non-specific binding sites. Membranes were then incubated overnight in 10 ml 5% milk-TBST containing 10 µl monoclonal antibodies against: PDK1 (Abcam, AB 47987, Cambridge, MA, USA); PDK 2 (Abgent, AP 7039b, San Diego, CA, USA); PDK 4 (Abgent, AP 7041b, San Diego, CA, USA);

COX IV (Mitosciences, MS 407, Eugene, OR, USA); E1α (Molecular Probes, A 21323, Eugene, OR, USA) and E2 (Molecular Probes, A 21325, Eugene, OR, USA) antibodies.

The membranes were washed with TBST, 3 times for 5 min each, and then incubated for 1 h in 10 ml 5% milk-TBST containing 0.5 µl goat anti-rabbit IgG (HRP-conjugated horseradish peroxidase (HRP), Santa Cruz Biotechnology, CA, USA) for PDK 2 and 4, bovine anti-goat IgG HRP (Santa Cruz Biotechnology, CA, USA) for PDK 1, anti-mouse HRP for COX IV, E1α and E2, as described by LeBlanc et. al., 2004. COX IV was used as a loading control. Membranes were again washed with TBST, 3 times 5 min each, and incubated for 1 min with extremely sensitive enhanced chemiluminescent substrate Chemiglow (Alpha Innotech, San Leandro, CA, USA) to develop the immunoblots. Light from the antibody-antigen complexes were visualized the use of Fluro Chem 5500 (Alpha Innotech, San Leandro, CA, USA). Relative densities were quantified (ImageJ, National Institute of Health) and results are expressed as the intensity of the band in arbitrary units.

Determination of Phosphagen Concentrations

Consistent with current literature, phosphagen concentrations were examined to determine muscle viability (Antolic et al. 2007). The extraction process was completed after the frozen EDL muscles were freeze-dried (Labconco Corporation; Kansas City, MO, USA). Once the water from the EDL muscles was completely removed, the tendons and connective tissue were teased apart from the muscle tissue, and then the muscle tissue was powdered. The powdered tissue was then weighed in a pre-weighed micro centrifuge tube and placed on ice. Once cold, 150-250 vol. of pre-cooled 0.5 M perchloric acid (PCA) was added to the powdered tissue and centrifuged (Beckman Coulter, Allegra 21; Palo Alto, CA, USA) at 4°C at 15 000 *g* for 10 min to precipitate debris and remove

enzymes that can influence the metabolite concentration. The supernatant was removed and potassium bicarbonate ($KHCO_3$) was added. Finally, the sample was centrifuged at 4°C at 15 000 *g* for 10 min and the supernatant was removed and stored at -80°C until assayed for phosphagens and lactate. Muscle phosphagen concentrations were determined by flurometric techniques according to procedures described by Harris et al (1974) and modified by Green et al. (1987). Each sample was analyzed in triplicate. For adenosine triphosphate (ATP) and phosphocreatine (PCr) concentration measurements a reagent containing 0.1 M Tris buffer (pH 8.1), 1M $MgCl_2$, 0.5 M DTT, 100 mM glucose, 50 mM nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate dehydrogenase (G-6-P-DH) (Sigma G-5760) was first added and analyzed at sensitivity of 80, excitation setting 340 and emission setting 460, with the Multi-Detection Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). The second and third reactions were initiated with the addition of hexokinase and then the addition of phosphocreatine kinase and adenosine diphosphate. Both were analyzed and read at the same previously stated settings. All measurements were made fluorometrically on a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at an excitation setting 340 nm and emission setting 460 nm, creating a baseline reading.

Statistics

PDHa activity was analyzed with SigmaStat, (Port Richmond, CA) as a two-way ANOVA (stimulation frequency; WT or KO). When significance was detected, means were compared using a Tukey's post hoc test. For protein contents and total PDK activity data, an unpaired two-tailed *t*-test was used. Significance was accepted at $p < 0.05$.

CHAPTER 5

RESULTS

Muscle Viability

Viability of muscle incubations was confirmed in our hands by measuring phosphagen concentrations (ATP and PCr) in a series of independent 30 min resting incubations (n=3). PCr was 80±2 and ATP was 25±2 mmol/kg dry muscle respectively.

PDHa Activity

As expected, there was a main effect for higher PDHa activity with muscle contraction for both WT and PDK2 KO. There was also a main effect for lower PDHa activity with contraction in the PDK2 KO muscles compared to WT [Fig. 7].

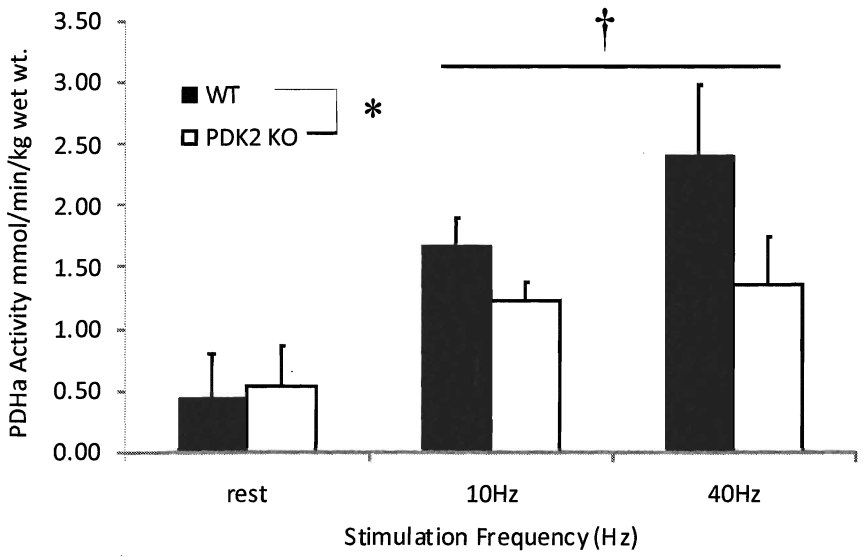

Figure 1. PDHa activity during rest, low and moderate intensity contraction in WT and PDK2 KO mice. PDHa activity was measured on whole muscle homogenates and is reported in units of mmol·min^{-1}·kg wet muscle^{-1}. *, main effect for lower PDHa activation in PDK2 KO muscles vs. WT, $p < 0.05$. †, main effect for stimulation to increase PDHa activation $p < 0.05$.

Total PDK Activity

Total PDH kinase activity was ~4-fold greater in WT mice in comparison to PDK2 KO ($p < 0.05$) [Fig. 8].

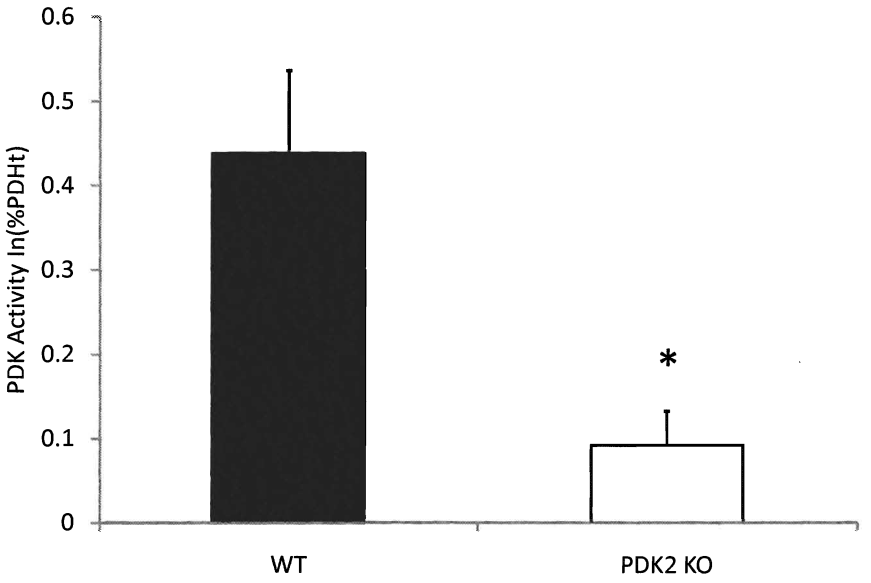

Figure 8. PDK activity in WT and PDK2 KO hindlimb muscle mitochondria. *PDK* activity was measured on isolated intact mitochondria and is reported as first-order rate constant for ATP-dependent inhibition of PDH_a (units are min^{-1}). n = 5 in each group.*Significantly different from WT, $p < 0.05$.

PDK1, 2 and 4 Isoform Content

Western blot analysis was used to determine the protein content of the three PDK isoforms, two PDH subunits E1-alpha and E2, and the mitochondrial marker COXIV in both WT and PDK2 KO. PDK2 protein was not detected by Western blot analysis in skeletal muscle extracts of PDK2 KO [Fig. 9]. The amount of PDK4 protein content was similar in both WT and PDK2 KO ($p = 0.19$) [Fig. 10], while PDK1 protein showed a 44% increase ($p < 0.05$) in the muscle mitochondria from PDK2 KO [Fig. 11].

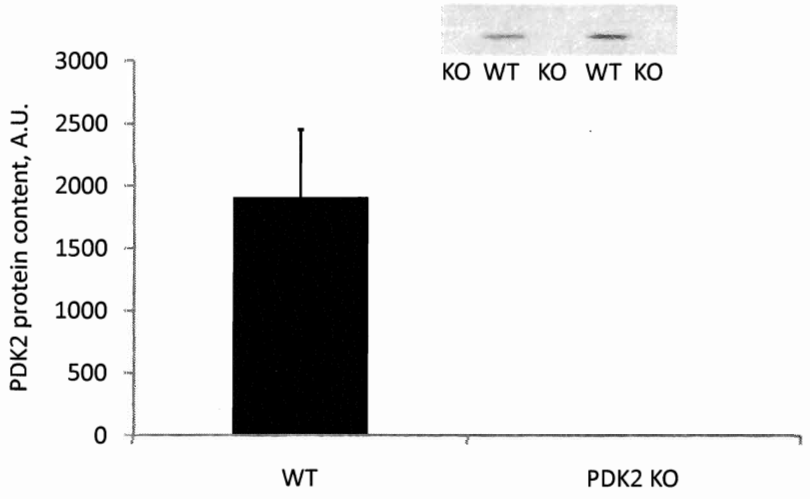

Figure 9. PDK2 protein content in WT and PDK2 KO mice. Inset, representative protein blots for WT (wild type) and PDK 2KO (PDK2 knockout) mice. Values are means ± S.E.M. with n = 6 in each group.

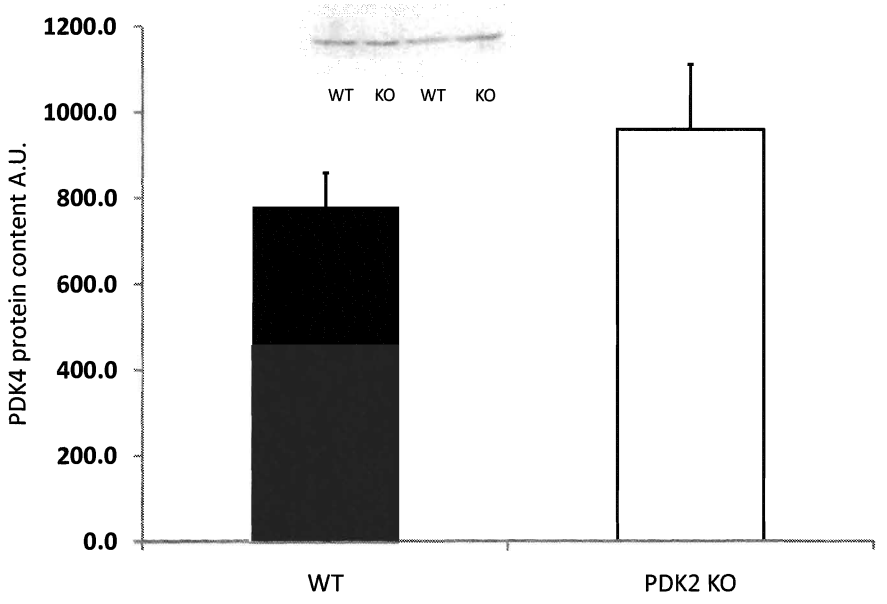

Figure 10. PDK4 protein content in WT and PDK2 KO mice. Inset, representative protein blots for WT (wild type) and 2KO (PDK2 knockout) mice. Values are means ± S.E.M. with n = 5 in each group, $p = 0.19$.

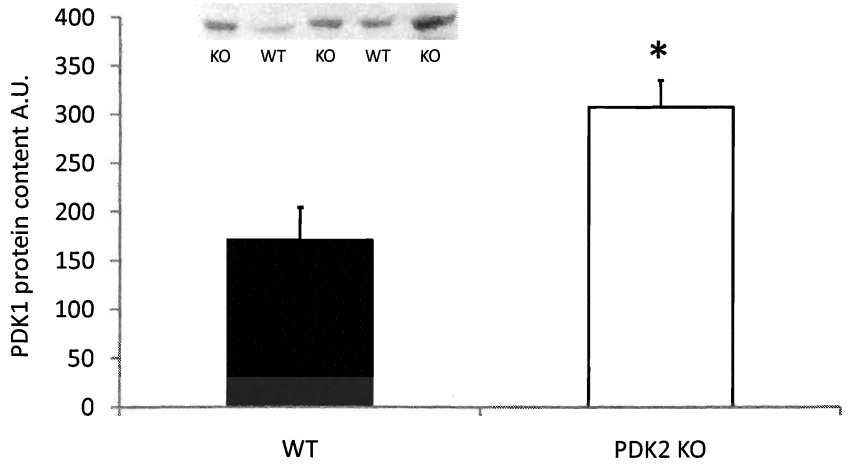

Figure 11. Representative Western blot of PDK1 protein content in WT and PDK2 KO mice. Inset, protein blots for WT (wild type) and 2KO (PDK2 knockout) mice. Values are means ± S.E.M. with n = 5 in each group.*Significantly different from WT, $p < 0.05$.

PDH complex subunits and COXIV content

The deletion of the PDK2 isoform also affected the protein content of E1-α protein showing a 32% increase ($p < 0.05$) [Fig. 12]. No significant difference was found in E2 protein content between WT and PDK2 KO mice, although there was a trend towards slightly increased E2 ($p = 0.112$) [Fig. 14]. This did not reflect a generalized change in mitochondrial oxidative capacity, since COXIV protein was not higher in PDK2 KOs ($p = 0.3301$) [Fig .13].

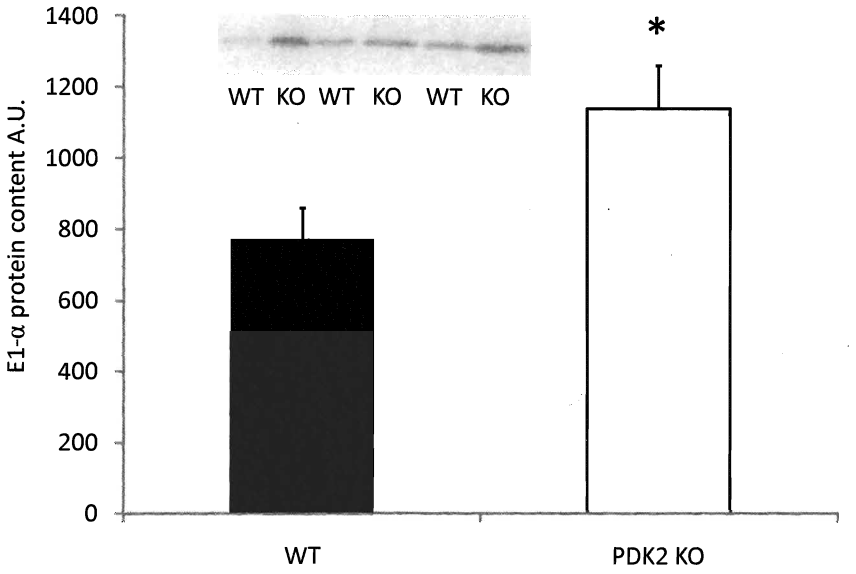

Figure 12. E1-α protein content in WT and PDK2 KO mice. Insert, protein blots for WT (wild type) and 2KO (PDK2 knockout) mice. Values are means ± S.E.M. with n = 5 in each group. *Significant difference from WT, $p < 0.05$.

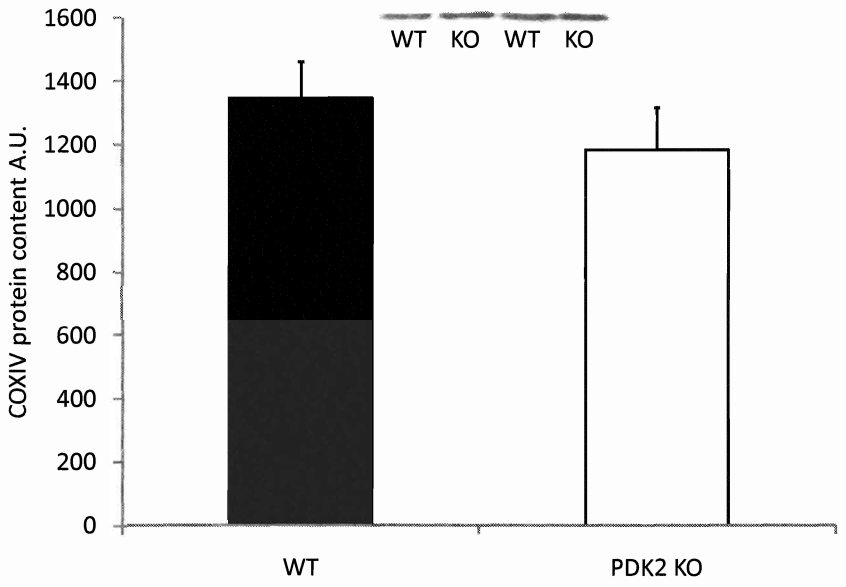

Figure 13. COXIV protein content in WT and PDK2 KO mice. Inset, representative protein blots for WT (wild type) and PDK 2KO (PDK2 knockout) mice. Values are means ± S.E.M. with n = 5 in each group, $p = 0.3301$.

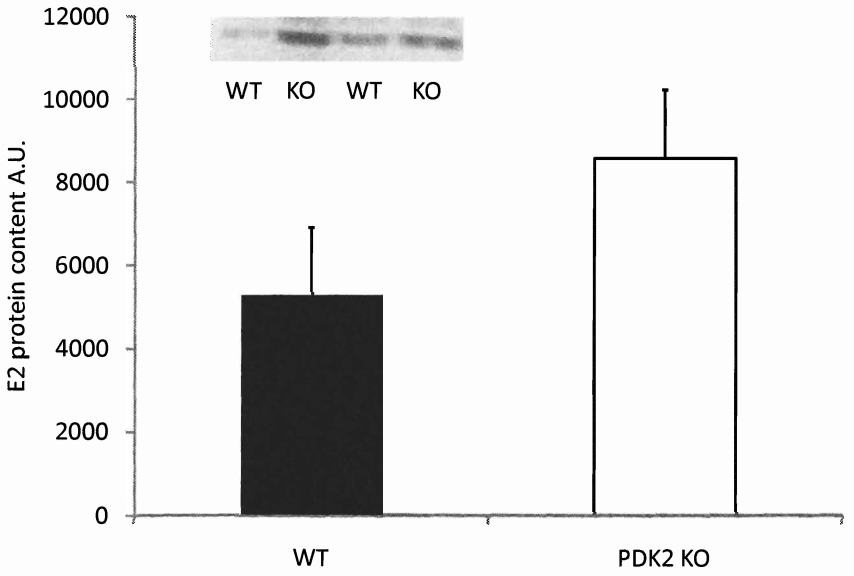

Figure 14. E2 protein content in WT and PDK2 KO mice. Inset, representative protein blots for WT (wild type) and PDK 2KO (PDK2 knockout) mice. Values are means ± S.E.M. with n = 5 in each group, $p = 0.112$.

CHAPTER 6

Discussion

This study describes the effects of the deletion of the PDK2 isoform in mice on PDHa activity during contraction, the relative expressions of the remaining PDKs and their total combined activity in skeletal muscle. As well, muscle expression of individual subunits of the PDH complex and the oxidative capacity of these genetically modified mice were examined. The major finding was that PDHa activity was lower during muscle contraction in the PDK2 KO mice compared to the WT mice, in spite of the fact that these mice lack the most abundant PDK isoform and have markedly lower total PDK activity. There was significantly increased expression of PDK1 protein (but not PDK4 protein) which could account for the decreased PDHa activity in PDK2 KO mice. In what appears to be an adaptive response, there was a 32% increase in E1-α protein expression in PDK2 KO mice, but this did not reflect a generalized increase in oxidative capacity.

Model Viability

The viability of the muscle incubations was confirmed through the analysis of phosphagen concentrations in frozen EDL muscles from separate 30 min incubations. In our hands, ATP and PCr values were similar to normal resting muscle values, confirming that at rest the viability was not compromised after the muscles were surgically removed, placed into the organ bath. EDL muscle ATP and PCr were found to be 25±2 and 80±2 mmol/kg dry muscle respectively, which is consistent with previous work in resting rat EDL muscle (ATP and PCr levels of ~ 30 and 77 mmol/kg dry muscle respectively; Antolic et al. 2007). The viability of the mouse EDL muscle incubation procedure has been previously well established (as reviewed by Bonen et al. 1994). For these types of

studies, mouse EDL muscle is an easily isolated fusiform muscle, and has a small diameter in mature animals (<1mm) therefore allowing consistent and rapid O_2 diffusion during incubation. Although the muscle has a small diameter, the temperature of the incubation is a major viability determinant. Specifically, at higher, more physiological temperatures (eg. 37°C) muscle oxygen consumption demand increases but the rate of O_2 diffusion does not increase proportionally (Bonen et al 1994). This is amplified during contraction by an additional increase in the rate of metabolism and O_2 consumption from the contracting muscle. Therefore, to counteract these potential problems and ensure a viable muscle, the muscle incubations were completed at 25°C at which temperature the diameter of the mouse EDL is well below the critical thickness necessary to support oxygen consumption. While the critical thickness of mouse EDL muscles for O_2 diffusion at higher temperatures is 1.94 mm (Bonen et al. 2007), the calculated maximum diffusion distance at 25°C is 0.3 mm (Barclay, 2005), and therefore O_2 diffusion would be capable of supporting the muscles metabolic rate during contraction.

For this study, both the mouse EDL and hindlimb were analyzed to determine the combined effect of the deletion of the PDK2 isoform had on PDHa and total PDK activity as well as the protein contents of the PDK isoforms and the PDH components during muscle contraction. Both of these muscles were used in this study because the small size of the EDL muscle only allowed for the measurement of PDHa activity, while the hindlimb was used for the protein determinations and total PDK activity. The fibre type composition of both the EDL and the hindlimb in the mouse are similar, with the EDL primarily composed of type IIa and IIb fibres with a small amount of type I fibres (Crow and Kushmerick, 1982) while the hindlimb is composed of primarily type IIb fibres, with a small amount of type IId and IIa fibres respectively (Hamalainen and Pette,

1992). Both muscle groups have a similar fibre type composition, with the EDL having slightly more fast glycolytic fibres while the hindlimb contains more fast oxidative fibres. Due to the similarity in fibre type composition of both the EDL and the hindlimb these results show that in both muscles there are similar generalized adaptations seen as a result of the deletion of PDK2.

PDHa and total PDK activity

Our results confirm, through Western blotting, that PDK2 KO mice did not have any PDK2 protein present in comparison to WT mice. PDK2 is the most abundant isoform in skeletal muscle and is highly sensitive to pyruvate inhibition but demonstrates a higher sensitivity to the energy status of the cell (Gudi et al. 1995, Popov, 1997, Bowker-Kinley et al. 1998). As such, PDK2 has been referred to as the "energy responsive" isoform because of its role in decreasing PDHa activity during sub-maximal exercise after aerobic training (LeBlanc et al. 2004). Therefore, it was not surprising that total PDK activity was found to be ~4 fold greater in WT than PDK2 KO.

The diaphragm of starved PDK4 KO mice was shown to have higher PDHa activity (Jeoung et al. 2006). These results are consistent with our findings that PDHa activity was higher at rest and both stimulation frequencies in PDK4 KO EDL muscles (Martin et al. unpublished). Therefore, given the lower total PDK activity, our hypothesis was that PDHa activity would be even higher in PDK2 KO mice. We believed that the removal of the PDK2 isoform would allow less phosphorylation of the PDH complex and result in a higher active component at a given stimulation intensity (Gudi et al., 1995, Sugden and Holeness, 2003). Predictably, a main effect for stimulation frequency (and higher contraction intensity) to increase PDHa activation was observed, which is

consistent with other work that generally shows that the activation state of the PDH complex parallels exercise intensity (Howlett et al. 1998 , Constantin-Teodosiu et al. 1991). However, quite surprisingly we also observed lower PDHa activation in PDK2 KO muscles in comparison to WT. This suggests that the activation of the complex during muscle contraction is more reliant on the collective kinetic properties of the population of the PDK isoforms than on the maximal total PDK activity available to down-regulate the complex.

The allosteric regulators of the PDH complex through PDP1 is Ca^{2+} and the PDKs are ATP/ADP, NADH/NAD^{+}, and acetyl-CoA/CoA ratios and pyruvate respectively. At a given stimulation frequency, the mitochondrial Ca^{2+} concentration would be similar, therefore the lower PDHa activation in PDK2 KO mice could be caused by the relative response of the remaining PDK isoforms to the present allosteric effectors. While both PDK1 and PDK4 are expressed in generally very low concentrations in skeletal muscle, they are hugely different in their responses to allosteric effectors, particularly energy charge, acetyl-CoA and pyruvate concentrations, which are changed during contraction. As such, it would be important to fully understand what changes, if any, were occurring in the expression of these two isoforms in the PDK2 KO mice.

PDK1 and PDK4 protein content

In muscle of PDK2 KO mice PDK1 protein content increased 44%, while PDK4 protein was unaltered. PDK4 has been shown to be upregulated during many dietary perturbations (Orfali et al. 1993, Wu et al. 1998, Wu et al. 1999) while PDK1 protein expression has remained unchanged throughout all physiological perturbations studied

thus far (Peters et al. 2001, Spriet et al. 2004., LeBlanc et al. 2004), although increased PDK1 has been observed recently during hypoxic conditions (Semenza, 2007). Even though the protein content of PDK4 remained unchanged, the exposure of the allosteric properties of PDK4 (particularly its insensitivity to pyruvate inhibition) could be a possible contributor to the lower PDHa activity observed during contraction in PDK2 KO mice.

To our knowledge, this the first study to report an increase in PDK1 content in the absence of hypoxia. PDK1 has a specific activity of about 30-50% lower than PDK3 (Bowker-Kinley et al. 1998), however, its activity is considerably higher than both those of PDK2 and PDK4. Of the four isoforms, both PDK1 and PDK2 were found to be about 70% similar in primary structure (Bowker-Kinley et al. 1998). PDK2 is known to be highly sensitive to pyruvate inhibition (Bowker-Kinley et al 1998; Sugden and Holness 2001), while PDK1 is even more sensitive to ADP inhibition than PDK4 (Bowker-Kinley et al 1998). PDK1 is also the only isoform that can phosphorylate site 3 of the E1-α subunit (Korotchkina et al. 1995). This is significant because PDHa activity of the complex can be sufficiently decreased or inhibited through the phosphorylation of only site 1, while the phosphorylation of sites 2 and 3 effectively cause the PDH complex to be harder to dephosphorylate or activate by the PDPs. Thus, the increase in PDK1 protein content could increase the phosphorylation of site 3, making it more difficult for the PDPs to activate the complex, and causing the lower PDHa activity in PDK2 KO muscle with the contraction stimuli. This underscores the importance of the kinetic properties of the population of isoforms rather than the total activity, since even with the absence of PDK2 and lower total PDK activity, PDHa activity is still lower in PDK2 KO mice.

These results can be explained by the kinetic properties of PDK4 and possibly even more by the increase in PDK1 content, since PDK1 would be a potent inhibitor of the complex.

PDH-E1-α and PDH-E2 protein content

Western blotting to detect the PDH components E1-α and E2 demonstrated that there was a 32% higher E1-α protein content in PDK2 KO muscle compared to WT, while E2 content was unchanged. The higher E1-α protein content without alterations in E2 is consistent with what has been previously observed in human skeletal muscle with endurance training (LeBlanc et al. 2004, Love et al. 2009, unpublished results). Our work is consistent with their suggestion that because the E1-α subunit is rate-limiting for the decarboxylation of pyruvate to acetyl-CoA, the main reaction within the complex, it would therefore adapt earlier to accommodate the need for increased oxidation, an idea which had been previously proposed for other tissues (Maury et al. 1995). In the case of skeletal muscle of PDK2 KO mice, higher E1-α protein seems to be a possible compensatory strategy to allow for improved PDHa activity by increasing the expression of this rate-limiting subunit. This is especially important in these PDK2 KO mice during exercise, since they appear to have increased difficulty activating or 'turning on' the complex even at low-to-moderate contraction stimuli.

The protein content of the E2 subunit was slightly, but not significantly higher in PDK2 KO mice, similar to what was observed in human skeletal muscle after 8 weeks of training (LeBlanc et al. 2004). This suggests that E2 could be undergoing a slower adaptive upregulation, following the increase in rate-limiting E1-α subunit. However, these adaptations to increase PDH activity through the upregulation of the PDH subunit do not reflect a generalized increase in muscle oxidative capacity, because COX IV (a

subunit of the cytochrome *c* oxidase enzyme of the electron transport chain) was unchanged in the PDK2 KO compared to the WT mice.

PDK1 expression in the PDK2 KOs – mediated by HIF-1?

The increased expression of PDK1 seen in this study has also been observed in hypoxic cultured mouse and human cells (Kim et al. 2006, Fukuda et al. 2007). Although our PDK2 KO mice are not deprived of oxygen, their expression profile is similar to what has been previously observed during hypoxia, and has been linked to hypoxia-inducible factor 1 (HIF-1), a transcription factor that appears to be responsible for 'balancing' redox in the electron transport chain. In hypoxia, there is a decrease in the amount of O_2 delivered to the ETC and, as a result, there has been an observed increase in PDK1, thought to cause a reduction of NADH influx into the ETC, while decreasing COXIV activity by shifting expression from. the first COX IV isoform to the second more productive COX IV isoform, thereby increaseing complex IV activity (as reviewed by Semenza, 2008). While there was not an observed change in COX IV protein in the PDK2 KO mice, it is possible that such as shift obscured by the fact that our antibody detected both COXIV isoforms. While it is not immediately clear how deleting the PDK2 isoform would stimulate the same transcription profile as that seen during hypoxia, it warrants further attention. It is possible that by deleting the most abundant isoform and by dropping total PDK activity so low, the muscle adapts to decrease the unregulated influx of carbohydrate-derived reducing equivalents to the ETC through the transcription factor HIF-1.

Future Studies

Future studies are required to fully explore the role of the PDK1 isoform, specifically during exercise. From this work, it might be suitable to term this isoform the "small but mighty" isoform, since relatively little of it seems to be very potent in changing PDH regulation due to its sensitivities to allosteric regulators that are changing during muscle contraction. It was observed in this study that in the absence of PDK2, PDK1 was upregulated in a possible compensatory strategy which potentially resulted in decreased PDHa activity during low and moderate intensity stimulation, but a thorough understanding of its normal role in the full complement of PDH kinases is yet to be understood.

Previous research on the PDK1 isoform has been severely limited primarily due to the very small relative concentration of this isoform in mixed muscle, and the complete lack of effect any nutritional or training perturbation has had on PDK1 protein content. PDK1 is known to only phosphorylate site 3 which makes it difficult to reactivate PDH. Phospho-specific antibodies could be used to fully understand the mechanism of site 3 phosphorylation on E1-α and subsequently understand PDK1 as well. The removal of the PDK2 isoform generated an increase in PDK1 protein leads to the obvious next step of PDK2 and PDK4 double KO mice. These double KO mice would allow for more specific and singular physiological research to examine the PDK1 isoform, by removing the two major kinases and observing PDK1 in isolation.

This study focussed primarily on the PDK isoforms and their roles in regulating PDH complex activity, however, the PDP isoforms are also a major component of PDH complex regulation. Specifically, how does PDP1, an isoform that has been shown to increase with exercise training, affect PDHa activity in the PDK2 KO mice at rest or

during acute contraction? Further characterization of the PDP isoforms in these PDK2 KO mice is needed to fully understand the effect of the removal of the PDK2 isoform and the subsequent lower PDHa activity.

Another major and unexpected finding that requires more attention is the potential link between the upregulation of PDK1 and the transcription factor HIF-1. Further work is necessary to examine differences in HIF-1 expression in PDK2 KO mice, and how these adaptive changes occur over time. It is also important to obtain a more specific antibody for COX IV that separates the two COX IV isoforms to determine whether there was a shift from COX IV-1 to COX IV-2 as seen during hypoxia.

To our knowledge, the individual PDH components have not been as extensively studied during nutritional and exercise perturbations, and the increase that was seen in this study in PDK2 KO mice, might have implications during other situations. More information regarding the potentially slower upregulation of the E2 subunit is needed. Is there an adaptive upregulation of the E2 subunit with the removal of the PDK2 isoform, and why is the increase in E2 so much slower in comparison to the E1-α subunit?

Summary and Perspectives

There are many aspects of fuel regulation and utilization that remain unknown. This study set out to examine the role that the PDK2 isoform played at rest and during low and moderate intensity contraction, and also observed that total PDK activity may not be as important in determining carbohydrate flux through oxidation during contraction. It seems that the overall kinetic and allosteric properties of the collective PDK isoform population have a greater influence on carbohydrate flux. This study removed the most abundant PDK isoform, thereby revealing the normal inhibitory role of

the other two isoforms on the PDH complex. Essentially, under normal circumstances PDK2 seems to play a role in 'fine-tuning' the activation of the complex because of its acute sensitivity to pyruvate inhibition. It 'dilutes' out the overly inhibitory properties of the other two isoforms. However, this study was also the first physiological evidence that PDK1 is actually a very potent inhibitor of the PDH complex, which raises questions as to why it would be present in skeletal muscle at all. However, PDK1 is more abundant in heart muscle (Bowker-Kinley et al. 1998) and in oxidative skeletal muscle (Peters et al. 2001) which are both tissues that have an increased reliance on fat. Therefore, PDK1 may play a role in promoting potent inhibition of the PDH complex, and thereby increasing fat oxidation in these tissues.

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Appendix

Pyruvate dehydrogenase kinase specialist properties
(Adapted from Bowker-Kinley 1998 and Sudgen 2003)

	PDK1	**PDK2**	**PDK3**	**PDK4**
Site specific phosphorylation	activity for site 3	highest activity towards site 1	lowest activity towards site 1	highest activity towards site 2
K_m for ATP (μM)	60 ± 5	10 ± 1 **highest affinity**	50 ± 5	65 ± 6
K_i for ADP (μM)	370 ± 20	120 ± 20 **sensitivity is similar to PDK4**	80 ± 10	100 ± 15
K_i for DCA (mM)	1.0 ± 0.2	0.2 ± 0.05 **more sensitive to DCA vs PDK4 and PDK1**	8.0 ± 1.0	0.5 ± 0.2
Effector Sensitivity NADH (% of control)	120%	130%	110%	190% **highest sensitivity**
NADH + acetyl-CoA (% of control)	180%	320% **highest sensitivity**	60%	150%
Effect of: **Starvation**	No change in protein or mRNA expression	No change	No change	**Increase** in protein and mRNA expression
High Fat Diet	No change	No change	No change	**Increase** in protein and mRNA expression

Chemically-Induced Diabetes	No change	No change	No change	**Increase** in protein and mRNA expression
Exercise	No change	**Increase** in protein and mRNA expression	No change	No change

Breakdown of SIGMA medium-199

Product Name	Medium-199
Product Number	M4530

TEST	**SPECIFICATION**
APPEARANCE	CLEAR SOLUTION
PH TEST	7.0-7.6
OSMOLALITY TEST	280-310 $mOsm \cdot kg^{-1}$
STERILITY BY USP	STERILE
ENDOTOXIN ASSAY	NMT 1EU/ML
GLUCOSE CONTENT	0.9-1.1 g^{-1}
CELL CULTURE TEST	PASS
EXPIRATION DATE	12 MONTHS

Components	M4350 [1X] $g \cdot L^{-1}$
Inorganic Salts	
$CaCl_2 \cdot 2H_2O$	0.265
$Fe(NO_3)_3 \cdot 9H_2O$	0.00072
$MgSO_4$ (anhyd)	0.9767
KCl	0.4
KH_2PO_4	--
Na Acetate (anhyd)	0.05
$NaHCO_3$	2.2
NaCl	6.8
Na_2HPO_4 (anhyd)	--
NaH_2PO_4 (anhyd)	0.122
Amino Acids	
DL-Alanine	0.05
L-Arginine HCl	0.07
DL-Aspartic Acid	0.06
L-Cysteine HCl H_2O	0.00011
L-Cystine.2HCl	0.026
DL-Glutamic Acid	0.1336
L-Glutamine	0.1
Glycine	0.05
L-Histidine HCl H_2O	0.02188
Hydroxy-L-Proline	0.01
DL-Isoleucine	0.04

DL-Leucine	0.12
L-Lysine·HCl	0.07
DL-Metthionine	0.03
DL-Phenylalanine	0.05
L-Proline	0.04
DL-Serine	0.05
DL-Threonine	0.06
DL-Tryptophan	0.02
L-Tyrosine 2Na·2H_2O	0.05766
DL-Valine	0.05

Vitamins

Ascorbic Acid·Na	0.000056
D-Biotin	0.00001
Calciferol	0.0001
Choline Chloride	0.0005
Folic Acid	0.00001
Menadione (sodium bisulfite)	0.000016
Myo-Inositol	0.00005
Niacinamide	0.000025
Nicotinic Acid	0.000025
p-Amino Benzoic Acid	0.00005
D-Pantothenic Acid·1/2Ca	0.00001
Pyridoxal·HCl	0.000025
Pydridoxine·HCl	0.000025
Retinol Acetate	0.00014
Riboflavin	0.00001
DL-α-Tocopherol Phosphate·Na	0.00001
Thiamine·HCl	0.00001

Other

Adenine Sulfate	0.01
Adenosine Triphosphate·2Na	0.001
Adenosine Monophosphate·Na	0.000238
Cholesterol	0.0002
Deoxyribose	0.0005
Glucose	0.1
Gluthathione (reduced)	0.00005
Guanine.HCl	0.0003
HEPES	--
Hypoxanthine	0.0003
Phenol Red·Na	0.0213
Tween 80	0.02
Ribose	0.0005
Thymine	0.0003

Uracil	0.0003
Xanthine.Na	0.000344

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